Essential role of NAT1/p97/DAP5 in embryonic differentiation and the retinoic acid pathway

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NAT1/p97/DAP5 is a newly identified protein that shares homology with the translation initiation factor eIF4G. Studies *in vitro* and in transfected cells indicated that NAT1 might suppress global translation, thereby repressing cellular proliferation. Here we studied the functions of NAT1 *in vivo* by disrupting its gene in mice. *NAT1$^{-/-}$* embryos died during gastrulation, indicating a crucial role for NAT1 in embryogenesis. Undifferentiated *NAT1$^{-/-}$* embryonic stem cells were normal in morphology, proliferation, global translation and gene expression profile. However, *NAT1$^{-/-}$* cells exhibited an impaired ability to differentiate: they were resistant to differentiation induced by retinoic acid, and teratomas derived from them consisted of undifferentiated and poorly differentiated tissues. The expression of retinoic acid-responsive genes, such as the cell-cycle inhibitor p21^{WAF1}, was selectively impaired in *NAT1$^{-/-}$* cells. Transcription from synthetic retinoic acid-responsive elements was also impaired. These data demonstrated that this translation initiation factor homolog controls specific gene expression pathways required for cellular differentiation.
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Introduction

NAT1, also known as p97 (Imataka *et al*., 1997), DAP5 (Levy-Strumpf *et al*., 1997) and eukaryotic translation initiation factor (eIF)4G2 (Shaughnessy *et al*., 1997), was identified as a candidate molecule responsible for oncogenesis caused by overexpression of the RNA editing enzyme APOBEC-1 (Yamanaka *et al*., 1997). RNA editing is a post-transcriptional modification that alters nucleotide sequences of RNA. Apolipoprotein B mRNA editing, which is the first example of RNA editing described in mammals, converts a single cytidine (nucleotide 6666) to uridine (Innerarity *et al*., 1996). This C to U modification is deamination catalyzed by APOBEC-1 and a complementary factor (Mehta *et al*., 2000). In the physiological situation, the specificity of APOBEC-1 is extremely high: the single nucleotide in the apolipoprotein B mRNA is the only known substrate. However, overexpression of APOBEC-1 in transgenic mice and rabbits leads to hepatic dysplasia and hepatocellular carcinoma (Yamanaka *et al*., 1995). Examination of these livers with a modified differential displays identified NAT1 as an aberrantly edited mRNA. The editing introduces multiple premature termination codons in the mRNA and virtually eliminates the NAT1 protein. We therefore hypothesized that the loss of the NAT1 function might contribute to the tumor formation.

NAT1 is homologous to the carboxyl two-thirds of eIF4G (Hentze, 1997; Morley *et al*., 1997; Gingras *et al*., 1999; Keiper *et al*., 1999). eIF4G is an essential adapter molecule that binds and coordinates initiation factors eIF4E, eIF4A and eIF3 (Thach, 1992; Merrick and Hershey, 1996), the protein kinase Mnk1 (Pyronnet *et al*., 1999) and poly(A)-binding protein (Imataka *et al*., 1998). eIF4G also binds to ribosomes through eIF3. In contrast, NAT1 binds to eIF4A (Imataka *et al*., 1997; Yamanaka *et al*., 1997), eIF3 (Imataka *et al*., 1997) and Mnk1 (Pyronnet *et al*., 1999), but not to eIF4E (Imataka *et al*., 1997; Yamanaka *et al*., 1997) or the poly(A)-binding protein (Imataka *et al*., 1998). Overexpression of NAT1 in cultured cells inhibits both cap-dependent and cap-independent translation initiation of reporter genes (Imataka *et al*., 1997; Yamanaka *et al*., 1997) and decreases total protein synthesis. Thus, these studies *in vitro* and in transfected cells suggest that NAT1 is a natural eIF4G decoy that represses translation globally.

In the last several years, important roles of translation control in cellular functions, including proliferation and tumorigenesis, have been recognized (Mathews *et al*., 1996). Overexpression of eIF4E or eIF4G in NIH 3T3 cells results in malignant transformation, suggesting that these initiation factors can function as oncogenes (De Benedetti and Rhoads, 1990; Lazaris-Karatzas *et al*., 1990; Fukuchi-Shimogori *et al*., 1997). This notion is further supported by the finding that eIF4E is amplified in a number of human cancers (De Benedetti and Harris, 1999), and eIF4G is amplified in squamous cell lung carcinomas (Brass *et al*., 1997). We therefore hypothesized that NAT1 suppresses cellular proliferation and tumorigenesis by inhibiting the function of eIF4E and eIF4G.

In this study, we disrupted the mouse *NAT1* gene in embryonic stem (ES) cells and generated *NAT1$^{-/-}$* mice to study its *in vivo* functions. Here we show that NAT1 is crucial for cellular differentiation rather than proliferation
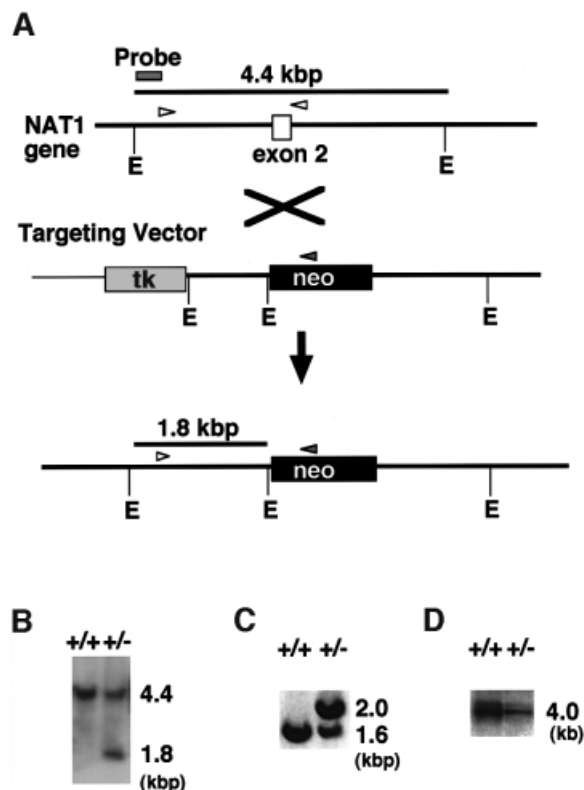
Fig. 1. Targeted disruption of the mouse *NAT1* locus. (**A**) Structures of the *NAT1* genomic locus, a targeting vector and the targeted locus generated by homologous recombination. Exon 2 of *NAT1* is shown as an open box. The targeting vector contains the *neo* gene (*neo*) in place of exon 2. In addition, the *pgk-tk* gene (*tk*) is attached to the 5′ end of the region of homology. The length of diagnostic *Eco*RI (E) restriction fragments and the location of a probe for Southern blot analysis are shown. Arrows indicate primers for PCR analysis. Figures are not drawn to scale. (**B**) Southern blot analysis. The targeting vector was introduced into RF8 ES cells, which were then selected with G418 and FIAU. Genomic DNA was isolated from surviving ES cell clones, digested with *Eco*RI and analyzed by Southern hybridization with the DNA probe indicated in (A). Specific hybridization produces a 4.4 kb band from the wild-type locus and a 1.8 kb band from the targeted locus. +/+ and +/– represent genotypes of *NAT1*⁺/⁺ and *NAT1*⁺/⁻ cells, respectively. (**C**) PCR analysis. PCR with three primers produces a 1.6 kb band from the wild-type locus and a 2.0 kb band from the targeted locus. (**D**) Northern blot analysis. Total RNA isolated from livers of each genotype as shown in (B) was electrophoresed, blotted and hybridized to a mouse cDNA probe including the entire sequence of the *NAT1* coding region.

Fig. 2. Characteristics of *NAT1*⁻/⁻ embryos. Embryos at E7.5 stained with hematoxylin and eosin (upper) and dissected embryos at E9.5 (lower). Normal (left) and abnormal (right) embryos are shown. By PCR, the abnormal embryo at E9.5 was found to have the *NAT1*⁻/⁻ genotype.

and that it functions as an essential determinant of specific gene expression pathways rather than a general suppressor of protein synthesis.

Results

Targeted disruption of the NAT1 gene in mouse ES cells and generation of NAT1⁺/⁻ mice

The targeting strategy used to disrupt the *NAT1* gene sequence in mouse ES cells is illustrated in Figure 1. The targeting vector was constructed by replacing exon 2 of the *NAT1* gene with the neomycin-resistance gene (*neo*) driven by the phosphoglycerate kinase gene (*pgk*) p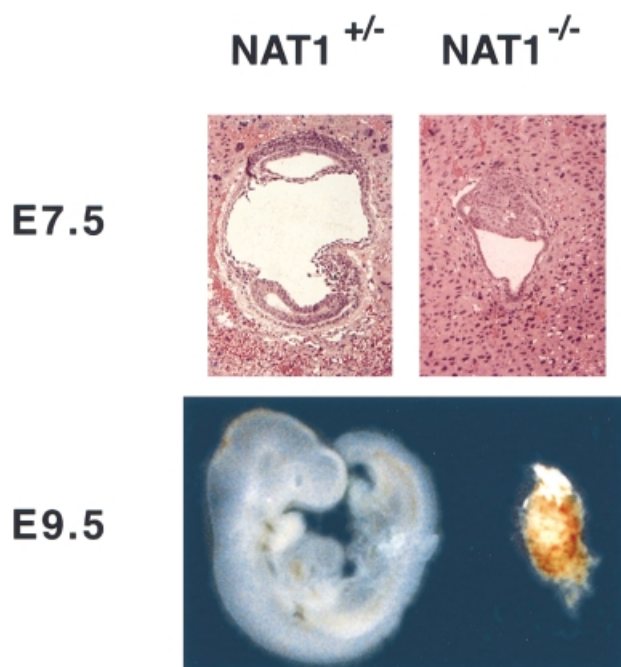romoter (Figure 1A). In addition, a copy of the *pgk* promoter–thymidine kinase (*tk*) gene was placed at the 5′ end of the construct for negative selection. Clones with the correct homologous recombination were identified by Southern blot analysis with a 5′ flanking probe (Figure 1B). *Eco*RI digestion of wild-type and targeted *NAT1* loci gave rise to 4.4 and 1.8 kb bands, respectively. The genotype of positive clones was confirmed further by PCR, which produces a 1.6 kb fragment for the wild-type locus and a 2 kb fragment for the targeted locus (Figure 1C). Male chimeric mice generated with a correctly targeted ES clone transmitted the *NAT1* gene mutation through the germline when mated with C57BL/6 female mice. Northern blot analysis of hepatic total RNA demonstrated 50% lower *NAT1* mRNA levels in *NAT1*⁺/⁻ mice than in wild-type littermates (Figure 1D). Heterozygous mice did not show any detectable phenotypic changes, including a higher incidence of tumor formation.

Null mutation of NAT1 causes embryonic lethality

To obtain *NAT1*⁻/⁻ mice, we intercrossed *NAT1*⁺/⁻ mice and determined the genotype of the resulting offspring by PCR. However, we did not obtain any homozygous mice from >20 heterozygous intercrosses. Analysis of embryos revealed that ~20% of the embryos between embryonic day (E)7.5 and E10.5 were poorly developed (Figure 2). Genotyping by PCR showed that these malformed embryos were *NAT1*⁻/⁻ (data not shown). At E11.5, *NAT1*⁻/⁻ embryos were absent, and 16.7% of deciduae (3/18) were undergoing resorption. Histological examination showed that 20.5% of E7.5 embryos (7/34) did not develop organized three primary germ layers (Figure 2). In these embryos, the trophectoderm and primitive endoderm developed, but the mesoderm did not emerge from the
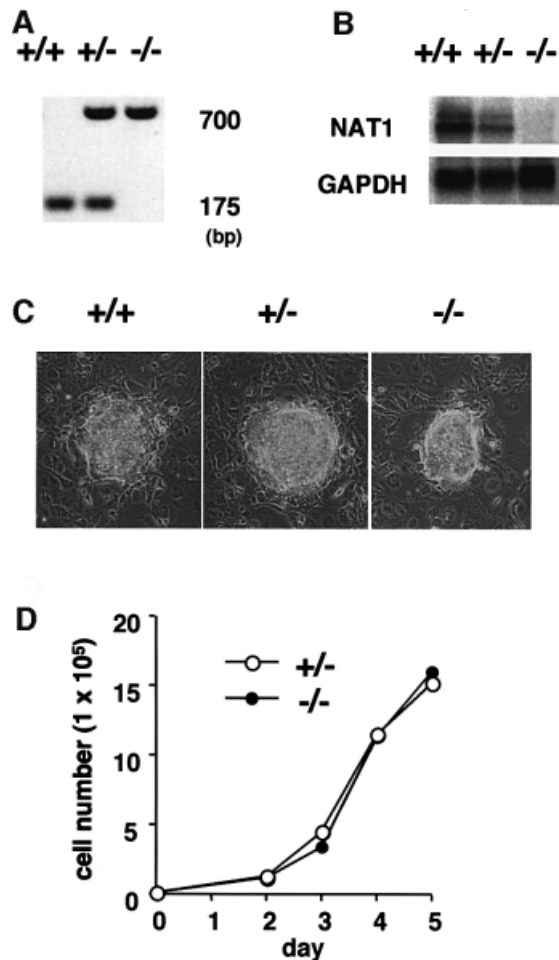
Fig. 4. Translational efficiency in *NAT1⁻/⁻* ES cells. (**A**) Total protein synthesis of three *NAT1⁺/⁻* clones (open column) and three *NAT1⁻/⁻* clones (closed column) was determined by the incorporation of [³⁵S]methionine and [³⁵S]cysteine into protein. (**B**) Bicistronic reporter genes. Two cDNAs were transcribed from a single *tk* promoter. The first, *Renilla* luciferase (*RLuc*) cDNA, is translated by a cap-dependent initiation, whereas the second, firefly luciferase (*FLuc*) cDNA, is translated from the IRESs of either EMCV, human c-*myc* or human *NAT1*. The late SV40 polyadenylation signal [poly(A)] is positioned 3′ to the *FLuc* gene to increase the expression level. (**C**) These reporter genes were introduced into three *NAT1⁺/⁻* clones and three *NAT1⁻/⁻* clones. Twenty-four hours later, IRES-dependent translation activity was estimated with *FLuc* activity normalized with *RLuc* activity.

Fig. 3. Characteristics of *NAT1⁻/⁻* ES cells. (**A**) PCR analyses showing the genotype of the *NAT1⁺/⁺*, *NAT1⁺/⁻* and *NAT1⁻/⁻* ES cell clones. (**B**) Northern blot analyses showing the *NAT1* mRNA level and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**C**) Morphology of ES cell colonies cultured on STO cells. (**D**) Proliferation of *NAT1⁺/⁻* and *NAT1⁻/⁻* cells. Ten thousand cells of three *NAT1⁺/⁻* and three *NAT1⁻/⁻* clones were maintained in the undifferentiated state on STO cells. Cell number was determined daily with a Coulter counter for 5 days. Shown are the averages of three clones in each group.

epiblast. These results showed that NAT1 is essential for gastrulation.

Normal growth in undifferentiated NAT1⁻/⁻ ES cells

To understand better the function of NAT1, we generated *NAT1⁻/⁻* ES cell clones from *NAT1⁺/⁻* cells by selection with high concentrations of G418 (Mortensen *et al.*, 1992). Of the 64 colonies that survived the selection, 29 were *NAT1⁻/⁻* by PCR (Figure 3A) and Southern blot analyses (data not shown). Northern blot analysis showed that the expression of *NAT1* mRNA was absent in *NAT1⁻/⁻* ES cells (Figure 3B).

When cultured on STO feeder cells that prevent differentiation of ES cells, *NAT1⁻/⁻* cells were morphologically indistinguishable from wild-type ES cells and *NAT1⁺/⁻* cells that had survived the same G418 selection (Figure 3C). In addition, *NAT1⁻/⁻* cells proliferated
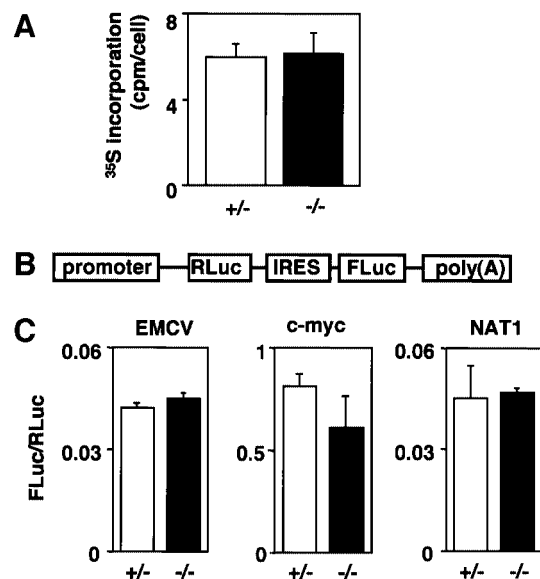
normally when grown on STO cells (Figure 3D), suggesting that NAT1 is not necessary for the growth of undifferentiated ES cells.

Normal translation in NAT1⁻/⁻ ES cells

Next, we investigated the effect of the *NAT1* disruption on total protein synthesis using [³⁵S]methionine and [³⁵S]cysteine incorporation. Both *NAT1⁺/⁻* and *NAT1⁻/⁻* ES cells showed similar amounts of incorporation of radioactive amino acids into proteins, suggesting that NAT1 is not involved in the regulation of general protein synthesis (Figure 4A).

We also examined translation from internal ribosome entry sites (IRESs), since NAT1 may be involved in cap-independent translation initiation from IRESs of cellular RNA, including *NAT1* itself (Henis-Korenblit *et al.*, 2000). We constructed three different reporter genes (ptk-DLuc-EMCV, ptk-DLuc-myc and ptk-DLuc-NAT1), in which two cDNAs are transcribed from the single *tk* promoter. The first cDNA, *Renilla* luciferase (*RLuc*), is translated with cap-dependent initiation, whereas the second cDNA, firefly luciferase (*FLuc*), is translated from either IRES of encephalomyocarditis virus (EMCV), the IRES of human c-*myc* or the IRES of human *NAT1* (Figure 4B). These reporter genes were transfected into *NAT1⁺/⁻* and *NAT1⁻/⁻* cells. The IRES activity of each construct was estimated by *FLuc* activity normalized by *RLuc* activity. The c-*myc* IRES was the most potent translation initiator (Figure 4C, c-*myc*); however, the activities of all three IRESs were
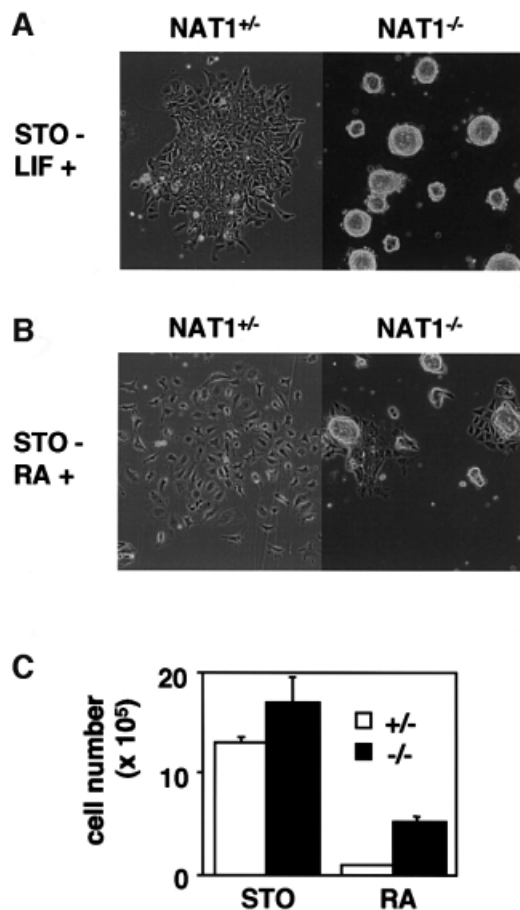
Fig. 5. Impaired differentiation in *NAT1*$^{-/-}$ ES cells. (**A**) Morphology of ES cells cultured without STO cells and treated with leukemia inhibitory factor (LIF, 1000 U/μl). (**B**) Morphology of ES cells treated with RA (3 × 10^{-7} M) for 4 days. (**C**) Proliferation of ES cells. Ten thousand cells of three *NAT1*$^{+/-}$ and three *NAT1*$^{-/-}$ clones were either maintained undifferentiated on STO cells or induced to differentiate with RA. After 5 days, cell numbers were measured. Shown are the averages and the standard deviations of three clones. Experiments were repeated four times with consistent results.

indistinguishable in *NAT1*$^{+/-}$ and *NAT1*$^{-/-}$ cells. Thus, NAT1 is not necessary for cap-independent translation initiation mediated by the IRES of EMCV, c-*myc* and *NAT1* itself.

Impaired differentiation in NAT1$^{-/-}$ ES cells

In contrast to their normal growth and translation in the undifferentiated state, *NAT1*$^{-/-}$ ES cells exhibited signs of impaired differentiation. We first noticed this when we cultured cells without STO feeder cells to isolate DNA for PCR genotyping. Without the feeder cells, normal ES cells flattened due to partial differentiation. However, the cells remained small and tightly associated in 29 of 64 clones that had survived the high concentrations of G418 (Figure 5A). PCR analysis revealed that all of these 29 clones were *NAT1*$^{-/-}$, whereas the remaining clones were *NAT1*$^{+/-}$.

We hypothesized that the altered morphology of *NAT1*$^{-/-}$ cells might be attributable to their impaired ability to differentiate. To confirm this possibility, we examined the effect of all-*trans* retinoic acid (RA), a

potent inducer of differentiation (Strickland and Mahdavi, 1978) in three *NAT1*$^{+/-}$ and three *NAT1*$^{-/-}$ ES clones that had survived the high G418 selection. When *NAT1*$^{+/-}$ cells were treated with RA (3 × 10^{-7} M), they spread out and enlarged (Figure 5B). Moreover, their proliferation rate slowed markedly (Figure 5C). In contrast, the RA-treated *NAT1*$^{-/-}$ cells remained for the most part small and highly associated (Figure 5B). The growth suppressive effect of RA was much smaller in *NAT1*$^{-/-}$ cells than in *NAT1*$^{+/-}$ cells (Figure 5C). Taken together, these data show that NAT1 is essential for ES cell differentiation.

Impaired differentiation in teratomas derived from NAT1$^{-/-}$ ES cells

To confirm further the role of NAT1 in ES cell differentiation, we subcutaneously injected *NAT1*$^{+/-}$ and *NAT1*$^{-/-}$ ES cells into nude mice. Transplantation of normal ES cells results in the formation of teratomas consisting of all three primary germ layers (Damjanov, 1978). *NAT1*$^{+/-}$ and *NAT1*$^{-/-}$ ES cells formed tumors of similar weight (0.71 ± 0.42 and 0.54 ± 0.35 g, respectively). However, histological sections of the tumors revealed marked differences (Figure 6). Teratomas from *NAT1*$^{+/-}$ cells contained well-differentiated tissues of three primary germ layers, including columnar epithelium (endoderm), neural tissue and squamous epithelium (ectoderm), and striated muscle, cartilage and bone (mesoderm). In contrast, tumors developed from *NAT1*$^{-/-}$ cells were composed mainly of undifferentiated cells and neuroectoderm that was characterized by rosette-like formations and abundant mitotic figures. Squamous epithelia existed, but with little keratinization. Columnar epithelia with lumen were also present, but lacked ciliary or mucous cells. Furthermore, no striated muscle or cartilage tissues were observed in any of 14 tumors derived from four independent *NAT1*$^{-/-}$ ES cell clones. These results showed that NAT1 is important for proper differentiation of all three primary germ layers from ES cells. In particular, NAT1 may be indispensable for the formation of striated muscle and cartilage.

Aberrant expression of RA-responsive genes in NAT1$^{-/-}$ ES cells

Analysis of the gene expression profiles confirmed the essential role of NAT1 in ES cell differentiation at a molecular level. Membrane-based cDNA arrays consisting of 588 genes showed that the expression profiles of *NAT1*$^{+/-}$ and *NAT1*$^{-/-}$ ES cells were similar when maintained undifferentiated on STO cells (Figure 7A, left). RA altered the expression of many genes in *NAT1*$^{+/-}$ cells (Figure 7A, center). The expression of 19 genes increased >3-fold after the RA treatment. In great contrast, the RA-induced changes in gene expression were much smaller in *NAT1*$^{-/-}$ ES cells (Figure 7A, right). None of the 588 genes was induced or suppressed >3-fold in the absence of NAT1.

To study the role of NAT1 in gene expression in more detail, we quantified gene expression levels by northern blot analyses. Nine genes [those encoding insulin growth factor (IGF)-2, keratin 19, tissue plasminogen activator (tPA), cyclin D2, collagen IV, PN1, c-*jun*, p21^{WAF1} and cathepsin D] were induced, and two genes (those encoding Oct3/4 and Sox2) were reduced >3-fold in *NAT1*$^{+/-}$ cells
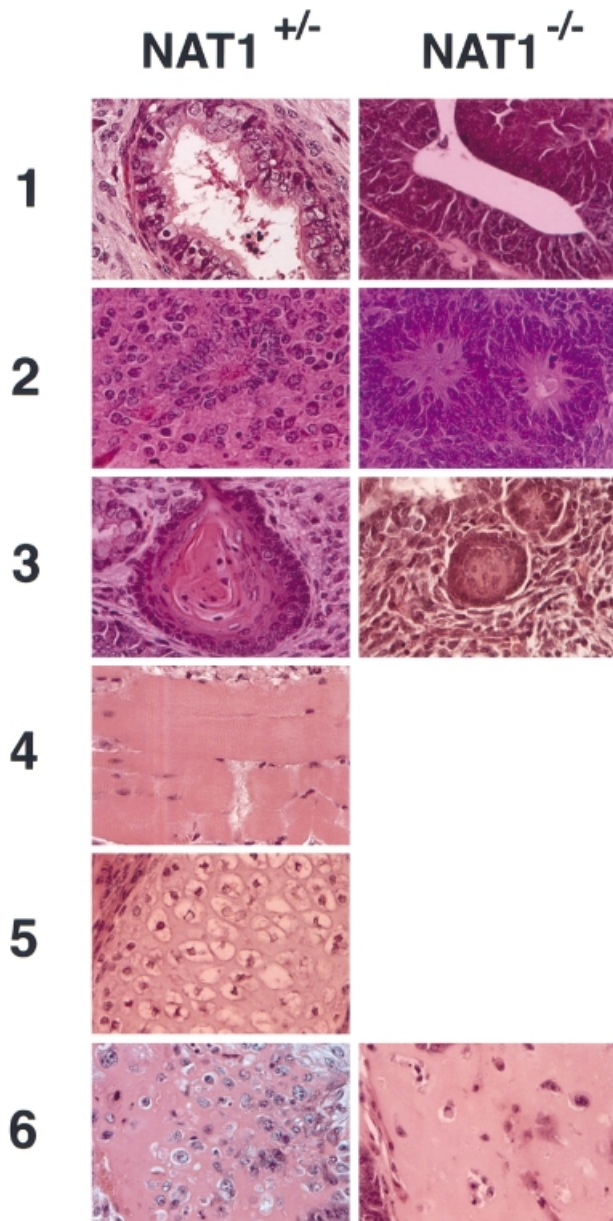
Fig. 6. Morphological examination of various tissues in teratomas derived from *NAT1*+/– (left) and *NAT1*–/– (right) ES cell clones: 1, columnar epithelium; 2, neural tissue; 3, squamous epithelium; 4, striated muscle; 5, cartilage; 6, bone. Striated muscle and cartilage were not observed in 14 tumors derived from four *NAT1*–/– clones. Sections were stained with hematoxylin and eosin.

Fig. 7. Altered gene expression in *NAT1*–/– ES cells. (**A**) cDNA arrays were used to compare the expression profiles of 588 genes between *NAT1*+/– and *NAT1*–/– ES cells, both of which were maintained undifferentiated on STO cells (left); between undifferentiated and differentiated *NAT1*+/– cells (center); and between undifferentiated and differentiated *NAT1*–/– cells (right). Differentiation was induced by RA treatment for 5 days. Total RNAs were isolated from three clones in each group and combined for comparison. Dashed lines indicate 2-fold differences between the two groups. Numbers shown are relative expression levels of each gene normalized with the sum of the expression levels of nine housekeeping genes (arbitrary units). (**B**) Northern blot analyses of gene expression. Genes in this figure were induced or reduced >3-fold by RA treatment in *NAT1*+/– cells. The expression level of each gene was normalized with the expression level in *NAT1*+/– cells grown on STO cells. Shown are the averages and the standard deviations of three clones in each genotype.

by RA treatment for 5 days (Figure 7B). In addition, the expression of *pax6*, a marker of neural differentiation, transiently increased 2 days after the initiation of RA treatment (data not shown).

In *NAT1*–/– ES cells, most of the RA-responsive genes were aberrantly expressed (Figure 7B). Both basal and RA-induced expression of *IGF-2* and *PN1* was impaired. The basal expression of the genes encoding keratin 19, tPA, cyclin D2, c-*jun*, p21*WAF1* and cathepsin D was normal but their RA-induced expression was impaired. The impaired induction of the cell-cycle inhibitor p21*WAF1* is likely to have contributed to the sustained proliferation of *NAT1*–/– cells after RA treatment. Repression of Oct3/4,

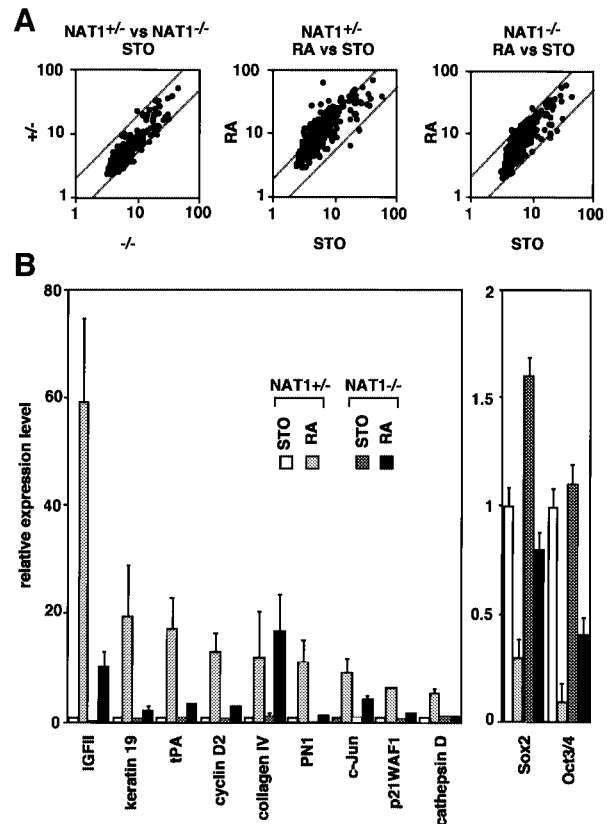the totipotent stem cell-specific transcription factor (Okamoto *et al.*, 1990; Nichols *et al.*, 1998), was also impaired. This suggests that NAT1 is involved in very early steps of ES cell differentiation. However, the induction of collagen IV was not significantly affected (Figure 7B), suggesting the existence of gene expression cascades independent of NAT1. The induction of *pax6* was also normal, which is consistent with the neural differentiation of *NAT1*–/– ES cells observed in teratomas. These results demonstrated that NAT1 is an important determinant of gene expression during RA-induced ES cell differentiation.

Impaired transcription from RA-responsive elements in *NAT1*–/– ES cells

To define further the role of NAT1 in RA-responsive gene expression, we used two reporter gene constructs in which the *FLuc* gene was transcribed from the minimum *tk*
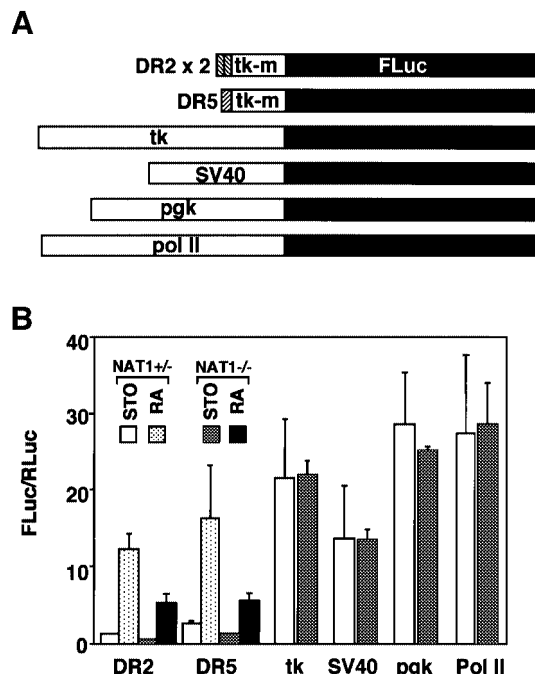
A

B

Fig. 8. Transcription of luciferase reporter genes containing RA-responsive elements in *NAT1*⁺ᐟ⁻ and *NAT1*⁻ᐟ⁻ ES cells. (**A**) We used two reporter genes containing either two copies of the RA-response element DR2, the minimum *tk* promoter (*tk-m*) and the *FLuc* gene or one copy of the RA-response element DR5, *tk-m* and *FLuc*. As controls, we used the *FLuc* reporter gene with the *tk*, SV40, *pgk* or polymerase II (*pol II*) promoter. (**B**) These plasmids together with pRL-TK, in which the *RLuc* gene is transcribed from the *tk* promoter, were introduced into three *NAT1*⁺ᐟ⁻ and three *NAT1*⁻ᐟ⁻ ES cell clones. The transfected cells were either maintained undifferentiated on STO cells or treated with RA (1×10^{-6} M). After 24 h, *FLuc* and *RLuc* activity was determined. The transfection efficiency was normalized with *RLuc* activity.

promoter ligated with either two copies of DR2 or one copy of DR5, both of which are RA-responsive elements (Ogura and Evans, 1995) (Figure 8A). *NAT1*⁺ᐟ⁻ and *NAT1*⁻ᐟ⁻ ES cells were transfected with these reporter genes and either cultured on STO cells or treated with RA for 24 h. An expression vector in which the *RLuc* gene was transcribed from the *tk* promoter was co-transfected to normalize transfection efficiency. In *NAT1*⁺ᐟ⁻ cells, RA stimulated the expression of both DR2 and DR5 constructs >10-fold (Figure 8B). In *NAT1*⁻ᐟ⁻ cells, both the basal and RA-induced expression of these constructs was significantly lower than in *NAT1*⁺ᐟ⁻ cells. As a control, we transfected cells with *FLuc* reporter genes driven by the *tk*, SV40, *pgk* or polymerase II (*pol II*) promoter. All of these control reporter genes showed similar *FLuc/RLuc* values in both *NAT1*⁺ᐟ⁻ and *NAT1*⁻ᐟ⁻ ES cells. These data suggest that transcription from the RA-responsive elements is selectively impaired in *NAT1*⁻ᐟ⁻ cells.

Discussion

This study demonstrates that NAT1 plays an essential role in embryogenesis and in the differentiation of ES cells, and is crucial for specific gene expression pathways associated with RA-induced differentiation. These *in vivo* functions of NAT1 revealed by gene targeting do not support conclusions based on the results of overexpressing NAT1

in transfected cells. Previous studies indicated that NAT1 represses translation in general, thereby suppressing cellular proliferation (Imataka *et al.*, 1997; Yamanaka *et al.*, 1997). In contrast, our data showed that the *NAT1* gene disruption did not affect total protein synthesis, suggesting that it is not involved in the regulation of general translation. This apparent discrepancy may be due to the abundant expression of eIF4A and eIF3 in cells (Duncan and Hershey, 1983; Duncan *et al.*, 1987). The normal expression level of endogenous NAT1 may not be high enough to sequester eIF4A and eIF3 sufficiently to alter translation. This is in contrast to 4E-binding proteins/PHAS-1, which inhibit translation by sequestering eIF4E, the least abundant of the translation initiation factors (Lin *et al.*, 1994; Pause *et al.*, 1994).

Another presumptive role of NAT1 is translation from IRESs (Levy-Strumpf *et al.*, 1997). This potential function is based on the sequence similarity between NAT1 and a C-terminal fragment of eIF4G that is produced by cleavage with several viral proteases (Etchison *et al.*, 1982; Devaney *et al.*, 1988). The cleavage divides eIF4G into an N-terminal fragment that binds to eIF4E and a C-terminal fragment that binds to eIF4A and eIF3–ribosomes (Lamphear *et al.*, 1995). As a result, cap-dependent translation initiation of cellular RNAs is disrupted. However, the C-terminal fragment can still initiate translation of viral RNAs from their IRES (Ohlmann *et al.*, 1996; Pestova *et al.*, 1996). Since NAT1 is homologous to the C-terminal fragment of eIF4G, it could possibly participate in IRES-dependent translation initiation. In fact, it was reported that NAT1 is involved in the IRES-dependent translation of *NAT1* mRNA itself (Henis-Korenblit *et al.*, 2000). However, we showed that IRESs from EMCV, c-*myc* and *NAT1* were as effective in *NAT1*⁻ᐟ⁻ ES cells as in *NAT1*⁺ᐟ⁻ cells, suggesting that NAT1 is not necessary or highly involved in the translation from these IRESs, at least in ES cells.

In contrast, we showed that NAT1 is essential for specific gene expression pathways associated with RA. The action of RA is mediated by transcriptional complexes consisting of RA receptors (RARs), retinoid X receptors (RXRs) and a number of co-regulators (Chambon, 1996). We found that RA responsiveness of direct target genes, such as *Oct3/4* (Pikarsky *et al.*, 1994) and p21^WAF1 (Kawasaki *et al.*, 1998), was impaired in *NAT1*⁻ᐟ⁻ ES cells. Furthermore, transcription from the synthetic RA-responsive elements, DR2 and DR5, was also impaired. These data suggest that NAT1 is required for the proper actions of the RA-dependent transcription complexes.

How does NAT1, a translation factor homolog, control transcription? Despite our results, the specific association with other translation initiation factors (Imataka *et al.*, 1997; Yamanaka *et al.*, 1997) and its cytoplasmic localization (our unpublished data) still argue that NAT1 is involved in translational control. One possible mechanism is that NAT1 controls translation of specific mRNAs. Long 5′ untranslated regions are often found in mRNAs encoding regulatory proteins like proto-oncogenes (van der Velden and Thomas, 1999). These regions contain three features that hamper ribosome scanning and cap-dependent initiation: structured regions, short open reading frames and IRESs (Willis, 1999). The mRNAs encoding one or more proteins in the RA-

dependent transcription complexes may possess these features and NAT1 may control their translation.

Although we could not show directly that loss of NAT1 function contributes to tumorigenesis, studies of two abnormal fusion proteins, PML–RARα and Tif1α–B-Raf, have demonstrated the importance of the RA pathway in hepatic oncogenesis (Zhong *et al*., 1999). The two oncoproteins, both of which are generated by chromosomal translocation, disrupt the RA pathway and cause hepatocellular carcinoma when expressed in mouse liver (Miki *et al*., 1991; David *et al*., 1997). The essential role of NAT1 in the RA pathway therefore supports the hypothesis that the aberrant editing of *NAT1* mRNA contributes to hepatocellular carcinoma caused by APOBEC-1. Further studies, such as the liver-specific gene knockout, will be required to determine whether NAT1, like many proteins with key roles in cellular differentiation, has tumor suppressor properties.

Three lines of evidence suggest that the role of NAT1 is not confined to the RA pathway. First, *NAT1*−/− ES cells were resistant to differentiation induced by culturing without STO feeder cells (RA-independent differentiation). Secondly, the phenotype of *NAT1*−/− embryos was more severe than that of embryos deficient in retinaldehyde dehydrogensae-2 (*Raldh2*), an enzyme responsible for embryonic RA synthesis; *NAT1*−/− embryos failed to undergo gastrulation, while *Raldh2*−/− embryos develop beyond gastrulation and die during midgestation (Niederreither *et al*., 1999, 2000). Thirdly, NAT1 was independently identified as a modulator of interferon-γ-mediated apoptosis (Levy-Strumpf *et al*., 1997). These data, taken together, suggest that NAT1 is involved not only in the RA pathway, but also in other cellular pathways important for development, differentiation and apoptosis.

In summary, our data demonstrate that NAT1 is crucial for early development, ES cell differentiation and the RA pathway, and suggest that this eIF4G homolog might control translation of specific mRNAs. Our data do not, however, exclude the possibility that NAT1 controls specific cellular pathways by a mechanism independent of translation. For example, NAT1 could bind to transcription factors and inhibit their nuclear import, thereby affecting specific transcription pathways. The precise biochemical mechanism of the selective role of NAT1 will be the focus of future investigations.

Materials and methods

Targeted disruption of the mouse NAT1 gene
A targeting vector with positive–negative selection was designed to delete exon 2, which contains the translation initiation codon of the mouse *NAT1* gene. A P1 bacteriophage clone containing the mouse *NAT1* gene was obtained from Genome Systems (St Louis, MO). A 1.2 kb fragment from intron 1 and a 5 kb fragment from intron 2 to exon 18 were amplified by PCR and used as the 5′ and 3′ homologous regions of the targeting vector. A *pgk-neo* minigene was ligated between the two DNA fragments, and a *pgk-tk* minigene was ligated upstream of the 5′ homologous region. The resulting targeting vector (pRTV-NAT1) was linearized with *Sal*I digestion and introduced into RF8 ES cells by electroporation (Meiner *et al*., 1996). Genomic DNAs from G418-resistant colonies were screened for homologous recombination by Southern blot and PCR analyses. For Southern blot analysis, genomic DNA was digested with *Eco*RI, separated on a 0.8% agarose gel and transferred to a nylon membrane. An 800 bp probe generated from exon 1 was labeled with [^{32}P]dCTP.

Hybridization with this probe produced a 4.4 kb band for the wild-type locus and a 1.8 kb band for the targeted locus. For PCR analysis, three primers were used. A single sense primer, S-U1 (CTTCCTCCC-CCTTCCCTCCCCCTTTT), was designed from exon 1 to amplify both wild-type and targeted loci, but not a targeting vector randomly integrated. The first antisense primer, L39 (TTTGTGTAGCCC-TGCCTGTCCTG), was designed from intron 2 to amplify the wild-type locus. The second antisense primer, S-L1 (CCTGCGTGC-AATCCATCTTGTTCAAT), was designed from the *neo* gene to amplify the targeted locus. PCR with these three primers produces a 1.6 kb fragment from the wild-type locus and a 2 kb fragment from the targeted locus. PCR was performed with the Expand Long Template PCR system (Boehringer Mannheim) according to the manufacturer's protocol except that 4% dimethylsulfoxide was added to the reaction.

Genotyping of mice, embryos and ES cells
After identifying the correctly targeted ES cell clone, we genotyped mice, embryos and ES cells by PCR with another set of primers. A single sense primer, S-U4 (CTGCAGTGCTGGGAGCGGAAATAAAT), was designed from intron 1 to amplify both wild-type and targeted loci. The first antisense primer, S-L4 (TTTGGCGGCTTGACAACGAAGAA-TCT), was designed from exon 2 to amplify the wild-type locus. The second antisense primer was S-L1 mentioned above. PCR with these three primers produced a 175 bp fragment from the wild-type locus and a 0.7 kb fragment from the targeted locus.

Generation of NAT1−/− ES cell clones
NAT1+/− ES cells (1 × 10^6 cells per 100 mm dish) were cultured with 1.5 mg/ml G418 (Mortensen *et al*., 1992). After 8 days, the genotype of the surviving colonies was determined by PCR.

Estimation of total protein synthesis
Total protein synthesis was estimated by [^{35}S]methionine and [^{35}S]cysteine incorporation. ES cells (1 × 10^4 cells per well of 24-well plates) from three *NAT1*+/− clones and three *NAT1*−/− clones were cultured on STO feeder cells. After 5 days, cells were washed twice with phosphate-buffered saline (PBS) and incubated with ES cell medium lacking methionine and cysteine. After 30 min, the medium was replaced with labeling medium containing [^{35}S]methionine and [^{35}S]cysteine (final concentration, 0.14 mCi/ml). After 3 h, cells were washed twice with PBS and dispersed with 100 μl of 2.5% trypsin solution, to which 400 μl of 0.1 mg/ml bovine serum albumin containing 0.02% NaN$_3$ was added. After cell numbers in 100 μl of this solution were counted with a Coulter counter, 400 μl of 20% trichloroacetic acid were added and mixed thoroughly. After a 1 h incubation on ice, the solution was mixed well, and 5 μl were applied to a glass fiber filter (FiltermatA, Wallac). The filter was washed twice with 10% trichloroacetic acid for 30 min and then with 100% ethanol for 30 min. The filter was completely dried, a solid scintillator (MeltiLex, Wallac) was applied onto the filter, and ^{35}S was measured with MicroBeta microplate scintillation counter (Wallac).

Analysis of IRES activity
Three bicistronic reporter constructs (Figure 4B, ptk-DLuc-EMCV, ptk-DLuc-myc and ptk-DLuc-NAT1) were constructed as follows. To make ptk-DLuc-EMCV, an *Xba*I fragment containing EMCV IRES and FLuc cDNA was isolated from pCAT-IRES-LUC and ligated into the *Xba*I site of pRL-TK. To make ptk-DLuc-myc, a portion of the human c-*myc* gene was amplified by PCR with primers MYC-U2320 (GGCTGAGGACCC-CCGAGCTGTGCT) and MYC-L4500 (GGGCCATGGTCGCGGGAG-GCTGCTGGAG). An *Xho*I–*Pvu*II fragment of the PCR product was ligated to *Xho*I–*Hin*dIII sites of pGV-BM2, in which an oligonucleotide containing *Xba*I and *Eco*RI sites had been incorporated in the *Kpn*I site of pGV-B (Promega). An *Xba*I fragment of the resulting plasmid, which contains IRES (myc)-FLuc, was ligated into the *Xba*I site of pRL-TK. To make ptk-DLuc-NAT1, the 5′ untranslated region of the human *NAT1* was amplified by PCR with primers NAT1-IRES-S (CGGAATTCA-GCAGTGAGTCGGAGCTCTATG) and NAT1-IRES-AS (ACTCTC-CATGGTGGCGGCTTGACAACGAAG). An *Eco*RI–*Nco*I fragment of the PCR product was ligated to *Eco*RI–*Nco*I sites of pGV-BM2. An *Xba*I fragment of the resulting plasmids, which contains IRES (NAT1)-FLuc, was ligated into the *Xba*I site of pRL-TK. These plasmids (10 μg of each) were introduced into ES cells (~1 × 10^7 cells) from three *NAT1*+/− clones and three *NAT1*−/− clones by electroporation. A quarter of the transfected cells of each clone were plated into a well of six-well plates with STO feeder cells. After 24 h, FLuc and RLuc activity was measured. IRES activity was estimated with FLuc activity normalized by RLuc activity.

Histological analysis of embryos and teratocarcinomas

Deciduae containing embryos were dissected from the uterus, fixed with 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. To produce teratomas in nude mice, 2×10^6 cells of three $NAT1^{+/-}$ and four $NAT1^{-/-}$ ES cell clones were injected subcutaneously into hind flanks. After 3 weeks, tumors were dissected, weighed and examined histologically as described above.

Analysis of gene expression

^{32}P-labeled cDNA probes were synthesized from total RNAs and hybridized to Atlas mouse cDNA arrays (Clontech) according to the manufacturer's protocol. The arrays were analyzed with a phosphoimager (Fuji, BAS2500). The expression level of each gene was normalized by the sum of nine housekeeping genes. Northern blot analyses were performed as described previously (Yamanaka *et al.*, 1997).

Transactivation assay of reporter genes

DR2-tk-LUC and DR5-tk-LUC constructs were kind gifts from Dr T.Ogura. To make control reporter genes, the following DNA fragments containing various promoters were ligated into appropriate cloning sites of pGV-B: an *Nhe*I–*Bgl*II fragment of pQBI-polII (Takara, Japan) containing the polymerase II promoter; an *Ssp*I–*Hind*III fragment of pKOV810 (Lexicon) containing the *pgk* promoter; and a *Bgl*II–*Hind*III fragment of pRL-TK (Promega) containing the *tk* promoter. These plasmids (10 μg of each) together with pRL-TK (0.3 μg) were introduced into ES cells (~1×10^7 cells) from three $NAT1^{+/-}$ clones and three $NAT1^{-/-}$ clones by electroporation. One-fourth of the transfected cells of each clone were plated into a well of six-well plates. Cells were either maintained undifferentiated on STO feeder cells or induced to differentiate with RA (1×10^6 M). After 24 h, FLuc and RLuc activity was measured. RLuc activity was used to normalize the transfection efficiency.

Acknowledgements

References

Brass,N., Heckel,D., Sahin,U., Pfreundschuh,M., Sybrecht,G.W. and Meese,E. (1997) Translation initiation factor eIF-4γ is encoded by an amplified gene and induces an immune response in squamous cell lung carcinoma. *Hum. Mol. Genet.*, **6**, 33–39.

Chambon,P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.*, **10**, 940–954.

Damjanov,I. (1978) Teratoma and teratocarcinoma in experimental animals. *Natl Cancer Inst. Monogr.*, **49**, 305–306.

David,G., Terris,B., Marchio,A., Lavau,C. and Dejean,A. (1997) The acute promyelocytic leukemia PML-RARα protein induces hepatic preneoplastic and neoplastic lesions in transgenic mice. *Oncogene*, **14**, 1547–1554.

De Benedetti,A. and Harris,A.L. (1999) eIF4E expression in tumors: its possible role in progression of malignancies. *Int. J. Biochem. Cell Biol.*, **31**, 59–72.

De Benedetti,A. and Rhoads,R.E. (1990) Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. *Proc. Natl Acad. Sci. USA*, **87**, 8212–8216.

Devaney,M.A., Vakharia,V.N., Lloyd,R.E., Ehrenfeld,E. and Grubman, M.J. (1988) Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *J. Virol.*, **62**, 4407–4409.

Duncan,R. and Hershey,J.W. (1983) Identification and quantitation of levels of protein synthesis initiation factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem.*, **258**, 7228–7235.

Duncan,R., Milburn,S.C. and Hershey,J.W. (1987) Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F

suggest a role in translational control. Heat shock effects on eIF-4F. *J. Biol. Chem.*, **262**, 380–388.

Etchison,D., Milburn,S.C., Edery,I., Sonenberg,N. and Hershey,J.W. (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.*, **257**, 14806–14810.

Fukuchi-Shimogori,T., Ishii,I., Kashiwagi,K., Mashiba,H., Ekimoto,H. and Igarashi,K. (1997) Malignant transformation by overproduction of translation initiation factor eIF4G. *Cancer Res.*, **57**, 5041–5044.

Gingras,A., Brian,B. and Sonenberg,N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.*, **68**, 913–963.

Henis-Korenblit,S., Strumpf,N.L., Goldstaub,D. and Kimchi,A. (2000) A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and internal ribosome entry site-mediated translation. *Mol. Cell. Biol.*, **20**, 496–506.

Hentze,M.W. (1997) eIF4G: a multipurpose ribosome adapter? *Science*, **275**, 500–501.

Imataka,H., Olsen,H.S. and Sonenberg,N. (1997) A new translational regulator with homology to eukaryotic translation initiation factor 4G. *EMBO J.*, **16**, 817–825.

Imataka,H., Gradi,A. and Sonenberg,N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.*, **17**, 7480–7489.

Innerarity,T.L., Boren,J., Yamanaka,S. and Olofsson,S.O. (1996) Biosynthesis of apolipoprotein B48-containing lipoproteins. Regulation by novel post-transcriptional mechanisms. *J. Biol. Chem.*, **271**, 2353–2356.

Kawasaki,H., Eckner,R., Yao,T.P., Taira,K., Chiu,R., Livingston,D.M. and Yokoyama,K.K. (1998) Distinct roles of the co-activators p300 and CBP in retinoic-acid-induced F9-cell differentiation. *Nature*, **393**, 284–289.

Keiper,B.D., Gan,W. and Rhoads,R.E. (1999) Protein synthesis initiation factor 4G. *Int. J. Biochem. Cell Biol.*, **31**, 37–41.

Lamphear,B.J., Kirchweger,R., Skern,T. and Rhoads,R.E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.*, **270**, 21975–21983.

Lazaris-Karatzas,A., Montine,K.S. and Sonenberg,N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. *Nature*, **345**, 544–547.

Levy-Strumpf,N., Deiss,L.P., Berissi,H. and Kimchi,A. (1997) DAP-5, a novel homolog of eukaryotic translation initiation factor 4G isolated as a putative modulator of γ interferon-induced programmed cell death. *Mol. Cell. Biol.*, **17**, 1615–1625.

Lin,T.A., Kong,X., Haystead,T.A., Pause,A., Belsham,G., Sonenberg,N. and Lawrence,J.C.,Jr (1994) PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science*, **266**, 653–656.

Mathews,M.B., Sonenberg,N. and Hershey,J.W.B. (1996) Origins and targets of translational control. In Hershey,J.W.B., Mathews,M.B. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–30.

Mehta,A., Kinter,M.T., Sherman,N.E. and Driscoll,D.M. (2000) Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Mol. Cell. Biol.*, **20**, 1846–1854.

Meiner,V.L. *et al.* (1996) Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc. Natl Acad. Sci. USA*, **93**, 14041–14046.

Merrick,W.C. and Hershey,J.W.B. (1996) The pathway and mechanism of eukaryotic protein synthesis. In Hershey,J.W.B., Mathews,M.B. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 31–70.

Miki,T., Fleming,T.P., Crescenzi,M., Molloy,C.J., Blam,S.B., Reynolds, S.H. and Aaronson,S.A. (1991) Development of a highly efficient expression cDNA cloning system: application to oncogene isolation. *Proc. Natl Acad. Sci. USA*, **88**, 5167–5171.

Morley,S.J., Curtis,P.S. and Pain,V.M. (1997) eIF4G: translation's mystery factor begins to yield its secrets. *RNA*, **3**, 1085–1104.

Mortensen,R.M., Conner,D.A., Chao,S., Geisterfer-Lowrance,A.A. and Seidman,J.G. (1992) Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.*, **12**, 2391–2395.

Nichols,J., Zevnik,B., Anastassiadis,K., Niwa,H., Klewe-Nebenius,D., Chambers,I., Scholer,H. and Smith,A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*, **95**, 379–391.

Niederreither,K., Subbarayan,V., Dolle,P. and Chambon,P. (1999) Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.*, **21**, 444–448.

Niederreither,K., Vermot,J., Schuhbaur,B., Chambon,P. and Dolle,P. (2000) Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development*, **127**, 75–85.

Ogura,T. and Evans,R.M. (1995) A retinoic acid-triggered cascade of HOXB1 gene activation. *Proc. Natl Acad. Sci. USA*, **92**, 387–391.

Ohlmann,T., Rau,M., Pain,V.M. and Morley,S.J. (1996) The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. *EMBO J.*, **15**, 1371–1382.

Okamoto,K., Okazawa,H., Okuda,A., Sakai,M., Muramatsu,M. and Hamada,H. (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell*, **60**, 461–472.

Pause,A., Belsham,G.J., Gingras,A.C., Donze,O., Lin,T.A., Lawrence, J.C.,Jr and Sonenberg,N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. *Nature*, **371**, 762–767.

Pestova,T.V., Shatsky,I.N. and Hellen,C.U. (1996) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell. Biol.*, **16**, 6870–6878.

Pikarsky,E., Sharir,H., Ben-Shushan,E. and Bergman,Y. (1994) Retinoic acid represses Oct-3/4 gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. *Mol. Cell. Biol.*, **14**, 1026–1038.

Pyronnet,S., Imataka,H., Gingras,A.C., Fukunaga,R., Hunter,T. and Sonenberg,N. (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *EMBO J.*, **18**, 270–279.

Shaughnessy,J.D.,Jr, Jenkins,N.A. and Copeland,N.G. (1997) cDNA cloning, expression analysis, and chromosomal localization of a gene with high homology to wheat eIF-(iso)4F and mammalian eIF-4G. *Genomics*, **39**, 192–197.

Strickland,S. and Mahdavi,V. (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell*, **15**, 393–403.

Thach,R.E. (1992) Cap recap: the involvement of eIF-4F in regulating gene expression. *Cell*, **68**, 177–180.

van der Velden,A.W. and Thomas,A.A. (1999) The role of the 5′ untranslated region of an mRNA in translation regulation during development. *Int. J. Biochem. Cell Biol.*, **31**, 87–106.

Willis,A.E. (1999) Translational control of growth factor and proto-oncogene expression. *Int. J. Biochem. Cell Biol.*, **31**, 73–86.

Yamanaka,S., Balestra,M.E., Ferrell,L.D., Fan,J., Arnold,K.S., Taylor,S., Taylor,J.M. and Innerarity,T.L. (1995) Apolipoprotein B mRNA-editing protein induces hepatocellular carcinoma and dysplasia in transgenic animals. *Proc. Natl Acad. Sci. USA*, **92**, 8483–8487.

Yamanaka,S., Poksay,K.S., Arnold,K.S. and Innerarity,T.L. (1997) A novel translational repressor mRNA is edited extensively in livers containing tumors caused by the transgene expression of the apoB mRNA-editing enzyme. *Genes Dev.*, **11**, 321–333.

Zhong,S., Delva,L., Rachez,C., Cenciarelli,C., Gandini,D., Zhang,H., Kalantry,S., Freedman,L.P. and Pandolfi,P.P. (1999) A RA-dependent, tumour-growth suppressive transcription complex is the target of the PML-RARα and T18 oncoproteins. *Nature Genet.*, **23**, 287–295.